

# UCSF

## UC San Francisco Previously Published Works

### Title

Conformational changes in the GTPase modules of the signal reception particle and its receptor drive initiation of protein translocation.

### Permalink

<https://escholarship.org/uc/item/2726v6dn>

### Journal

The Journal of cell biology, 178(4)

### ISSN

0021-9525

### Authors

Shan, Shu-ou  
Chandrasekar, Sowmya  
Walter, Peter

### Publication Date

2007-08-01

### DOI

10.1083/jcb.200702018

Peer reviewed

# Conformational changes in the GTPase modules of the signal reception particle and its receptor drive initiation of protein translocation

Shu-ou Shan,<sup>1</sup> Sowmya Chandrasekar,<sup>1</sup> and Peter Walter<sup>2</sup>

<sup>1</sup>Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125

<sup>2</sup>Howard Hughes Medical Institute and Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA 94158

**D**uring cotranslational protein targeting, two guanosine triphosphatase (GTPase) in the signal recognition particle (SRP) and its receptor (SR) form a unique complex in which hydrolyses of both guanosine triphosphates (GTP) are activated in a shared active site. It was thought that GTP hydrolysis drives the recycling of SRP and SR, but is not crucial for protein targeting. Here, we examined the translocation efficiency of mutant GTPases that block the interaction between SRP and SR at specific stages. Surprisingly, mutants that allow SRP–SR complex assembly but block GTPase activation severely

compromise protein translocation. These mutations map to the highly conserved insertion box domain loops that rearrange upon complex formation to form multiple catalytic interactions with the two GTPs. Thus, although GTP hydrolysis is not required, the molecular rearrangements that lead to GTPase activation are essential for protein targeting. Most importantly, our results show that an elaborate rearrangement within the SRP–SR GTPase complex is required to drive the unloading and initiate translocation of cargo proteins.

## Introduction

GTPases comprise a superfamily of proteins that provide molecular switches to regulate many cellular processes, including protein synthesis, signal transduction, cytoskeletal organization, vesicle transport, nuclear transport, spindle assembly, and many more (Gilman, 1987; Bourne et al., 1991; Mitin et al., 2005). The classic work on signaling GTPases, such as Ras, has established a “GTPase switch” paradigm in which a GTPase alternates between two distinct conformational and functional states: an active, GTP-bound state and an inactive, GDP-bound state. Both states are kinetically stable and are thus separated from one another temporally. Interconversion between the GTP- and GDP-bound states is facilitated by external regulatory factors, such as GTPase-activating proteins and guanine nucleotide exchange factors. This allows a GTPase to switch between “on” and “off” states in temporal succession in response to extra- or intracellular signaling cues.

Two homologous GTPases, one in the SRP54 subunit of the signal recognition particle (SRP) and one in the SRP receptor

(SR; called Ffh and FtsY in bacteria, respectively), mediate the cotranslational targeting of membrane and secretory proteins to the eukaryotic endoplasmic reticulum membrane, or the bacterial plasma membrane. During the targeting reaction, SRP and SR switch between different functional states (Walter and Johnson, 1994; Keenan et al., 2001). At the beginning of each targeting cycle, SRP binds to a nascent polypeptide that contains a signal sequence as it emerges from the ribosome (Walter et al., 1981; Pool et al., 2002). The ribosome–nascent chain complex (RNC) is then delivered to the membrane via an interaction between the SRP and SR. Upon arrival at the membrane, SRP releases its “cargo,” the RNC, to the translocation channel, or the translocon (Walter and Blobel, 1981; Gilmore et al., 1982a,b). Once the RNC is released, SRP and SR dissociate from each other, allowing another cycle of protein targeting to occur (Connolly and Gilmore, 1989; Connolly et al., 1991).

Analogous to other GTPases, the switches in the functional states of SRP and SR are coordinated by their GTPase cycles. Numerous biochemical experiments have shown that formation of a stable SRP–SR complex requires both GTPases to be bound with GTP or nonhydrolyzable GTP analogues (Connolly and Gilmore, 1993; Miller et al., 1993; Peluso et al., 2000); thus, GTP binding is critical for delivery of the cargo protein to the target membrane. However, less data are available

Correspondence to Shu-ou Shan: sshan@caltech.edu

Abbreviations used in this paper: GMPPNP, 5'-guanylylimido-diphosphate; IBD, insertion box domain; pPL, preprolactin; RNC, ribosome–nascent chain complex; SR, SRP receptor; SRP, signal recognition particle; TKRM, salt-washed and partial trypsin-digested microsomal membrane; WG, wheat germ.

The online version of this article contains supplemental material.

on the role of GTP hydrolysis in the protein targeting reaction. In the classical experiment by Wilson et al. (1988), it was shown that a nonhydrolyzable GTP analogue, 5'-guanylylimidodiphosphate (GMPPNP), can substitute for GTP and mediate a single round of protein translocation. However, in the presence of GMPPNP, the SRP and SR GTPases are irreversibly trapped in a stable complex and cannot mediate subsequent rounds of protein targeting (Connolly and Gilmore, 1989; Connolly et al., 1991). These observations led to the current model in which GTP hydrolysis is not important for the targeting reaction *per se*, but is used to drive the disassembly of the SRP–SR complex, thus regenerating free SRPs and SRs for subsequent rounds of protein targeting.

Nevertheless, this classical model might be an oversimplified picture, as the behavior of SRP and SR is modeled in analogy to the canonical GTPase switch mechanism, in which regulation is exerted by the switch of GTPases between the GTP- and GDP-bound states. However, the SRP and SR GTPases exhibit biochemical properties that are distinct from those of classical GTPases, and hence are likely to use different regulatory mechanisms. Unlike many other GTPases, both SRP and SR exhibit weak nucleotide affinities and fast nucleotide exchange rates (Jagath et al., 1998, 2000; Peluso et al., 2001), and thus do not require external nucleotide exchange factors. Furthermore, crystallographic analyses showed that the conformations of the SRP and SR GTPases are similar regardless of whether GTP or GDP is bound (Freymann et al., 1997, 1999; Montoya et al., 1997; Padmanabhan and Freymann, 2001); thus the exchange of GDP for GTP is unlikely to be the mechanism that switches these GTPases to the on state. In addition, SRP and SR reciprocally activate each other's GTPase activity once they form a complex with each other (Powers and Walter, 1995); thus regulation of GTP hydrolysis by an external GTPase-activating protein is unlikely to be the mechanism that turns these GTPases to the off state.

Instead, the recent crystal structure of the GTPase domains of the Ffh–FtsY complex showed that these GTPases undergo large-scale conformational changes only after they form a complex with one another when each GTPase is already bound with GTP (Egea et al., 2004; Focia et al., 2004). Compared with the structures of the apoproteins, two major conformational changes are observed upon complex formation. A major rearrangement occurs at the interface between the central GTPase G domain and the N domain, a unique insertion in the SRP subgroup of GTPases that packs tightly against the G domain. The readjustment of the relative position of the N and G domains allows the N domains of both proteins to bend toward its binding partner and form additional interface interactions with one another. The other major rearrangement occurs at the insertion box domain (IBD) loops of both GTPases. This loop is highly conserved in the SRP subfamily of GTPases but is not present in other GTPases. Upon complex formation, each IBD loop brings three key catalytic residues into the GTPase site of their respective protein to position and activate the nucleophilic water molecule and to stabilize the negative charges on the  $\gamma$ -phosphate.

Consistent with the crystal structure, multiple distinct classes of mutant GTPases have been isolated, each defective at a different step during the SRP–FtsY interaction (Shan and Walter, 2003;

Shan et al., 2004). Mutations of many residues at the N–G domain interface severely impair SRP–FtsY binding (class I), supporting the importance of this domain rearrangement for complex formation, as well as the importance of an extensive interaction surface that pays for the energetic cost of conformational changes during complex formation. Surprisingly, even after a stable complex is formed, single mutations in FtsY can block the reciprocal activation of GTP hydrolysis in both active sites (class II or “activation-defective” mutants). Thus, activation requires additional conformational changes across the interface that coordinate the positioning of catalytic residues and that are highly coupled between the two GTPase sites. Most of these mutations map to the IBD loop, supporting the importance of this loop in GTPase activation. A distinct class of mutants exhibit half-site reactivity and allows us to further uncouple the activation of the individual sites (class IV or “half-site” mutants). These mutants suggest the presence of additional conformational changes that complete the individual active sites.

These distinct classes of mutant GTPases strongly suggest that the SRP–FtsY interaction is a dynamic process involving multiple, discrete conformational changes that culminate in the activation of GTP hydrolysis. These results also raise the intriguing possibility that instead of using external regulatory factors, the conformational rearrangements during SRP–SR complex formation and activation may provide critical points for regulation during the protein targeting reaction (Shan et al., 2004). To test this notion, we have examined the effect of the different classes of mutant GTPases on the protein targeting reaction. Surprisingly, the class II, or activation-defective, mutants severely block protein translocation, even though assembly of the SRP–SR complex is unimpaired in these mutants. Thus the activation of GTP hydrolysis in the SRP–SR complex plays a much more important role in the protein targeting reaction than was previously thought.

## Results

### Development and calibration of a semiquantitative, cotranslational protein-targeting assay

To test the effect of mutant GTPases on the protein targeting reaction, we first developed an assay that reports on the efficiency of translocation by the bacterial SRP and SR (FtsY). Most of the existing assays in the bacterial system are qualitative, relying either on protease protection of the protein substrate by membrane vesicles after translocation (Koch et al., 1999, 2002) or on the ability of the protein substrate to cross-link to the SRP or to the sec translocon (Valent et al., 1995, 1998). In contrast, a much more robust translocation assay exists for eukaryotic systems, because translocation of a eukaryotic protein across the ER microsomal membrane results in efficient cleavage of the signal sequence by signal peptidase, allowing pre- and mature proteins to be resolved by SDS-PAGE, so that both reaction substrates and products can be visualized and the fraction of translocated protein can be quantitated. A similar assay does not yet exist for bacterial systems because most of the substrates for bacterial SRP are inner membrane proteins whose signal sequences are not cleaved upon translocation.

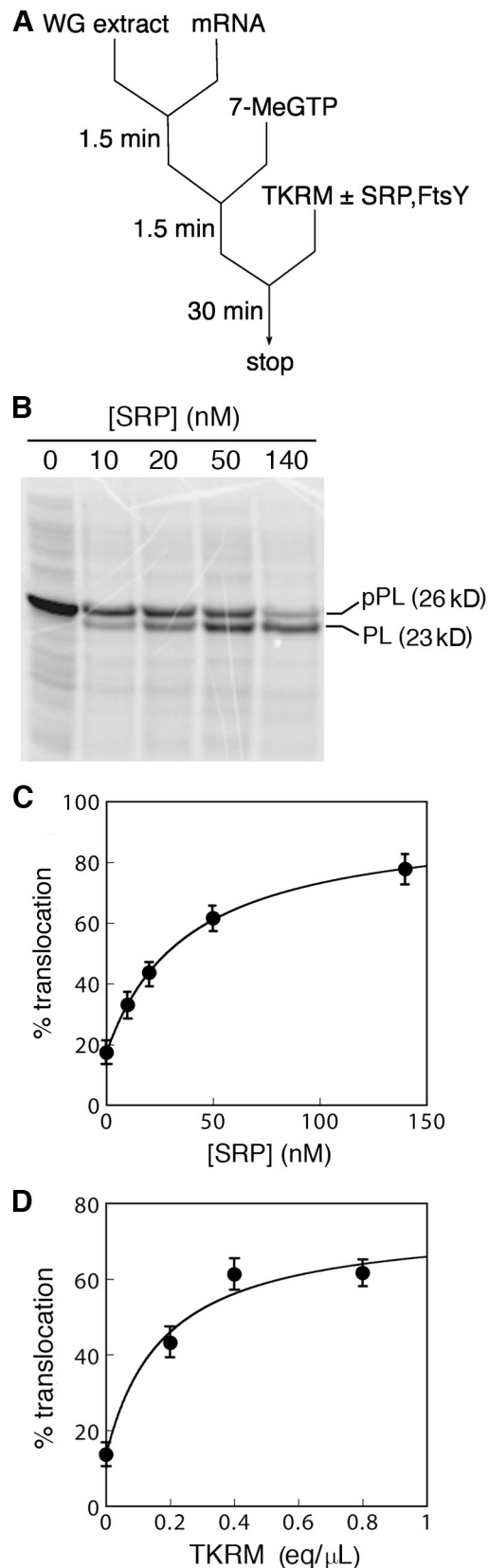


Figure 1. **A cotranslational assay to measure protein translocation by bacterial SRP and FtsY.** (A) Scheme for the cotranslational targeting assay. (B) SDS-PAGE analysis of the translocation of  $^{35}\text{S}$ -labeled pPL. pPL and prolactin indicate the precursor and signal sequence-cleaved form of prolactin, respectively. (C and D) Translocation efficiency is dependent on the

For these reasons, we decided to use a heterologous protein translocation assay pioneered by Powers and Walter (1997). In this assay, wheat germ (WG) translation extract is used to synthesize a mammalian SRP substrate, preprolactin (pPL). We then assessed the ability of bacterial SRP and FtsY to deliver pPL to microsomal membranes in which endogenous SRP and SR have been removed by TKRM (a high salt wash and partial trypsin digestion). To best mimic the in vivo targeting reaction, we designed the assay to report on translocation cotranslationally (Fig. 1 A). Shortly after translation is initiated, a cap analogue, 7-methyl-GTP, is added to inhibit additional rounds of translation initiation, such that translocation of only the first round of translation product is followed. *Escherichia coli* SRP (the Ffh protein bound to the 4.5S SRP RNA), FtsY, and TKRM are added to allow translocation of nascent pPL. Translation is continued for 20–30 min to allow completion of pPL synthesis, at which time the reaction is stopped and analyzed. Consistent with previous results (Powers and Walter, 1997), translocation of pPL is very robust in this heterologous system (Fig. 1 B) and depends on the concentration of *E. coli* SRP, FtsY, and TKRM (Fig. 1, B–D; and see Figs. 2, 3, and 5).

To probe the sensitivity and dynamic range of this targeting assay, we tested the translocation efficiency of mutant FtsY GTPases that block SRP–FtsY binding and therefore are expected to compromise the delivery of pPL to the membrane (Table I, class I mutants). Three FtsY mutants were tested. FtsY E475K specifically compromises SRP–FtsY binding by  $\sim 20$ -fold, but still allows efficient activation of GTP hydrolysis when complexes with SRP form at high protein concentrations (Shan et al., 2004). FtsY K399A has a more severe defect, compromising SRP–FtsY complex formation by 30-fold (Shan et al., 2004). In contrast, FtsY T307A blocks both complex formation and GTPase activation by  $>200$ -fold, as Thr307 is one of the key residues that coordinate the  $\text{Mg}^{2+}$  ion in the GTPase active site (Shan et al., 2004). As expected, mutant FtsY T307A almost completely blocks translocation of pPL (Fig. 2 and Fig. S3 A, squares, available at <http://www.jcb.org/cgi/content/full/jcb.200702018/DC1>), whereas mutants FtsY E475K and FtsY K399A reduce the translocation efficiency more modestly (two- and threefold, respectively; Fig. 2 and Fig. S3 A, triangles and closed circles). Other class I FtsY mutants that compromise SRP–FtsY binding by more moderate amounts (three- to fivefold) do not show a considerable translocation defect (not depicted). Thus we conclude that this assay can reliably detect translocation defects if SRP–SR complex formation is weakened by  $>20$ -fold. In contrast, more moderate defects are masked, presumably because targeting and translocation of preproteins occur much faster than protein synthesis (Fig. S1) and only become rate-limiting when the translocation efficiency is compromised beyond a certain threshold. Nevertheless, the assay reliably detects defects of GTPase mutations that substantially compromise the efficiency of protein targeting.

concentration of SRP (C) and membrane (D). The data represent the mean of two to three measurements, and the error bars represent the range of values observed.

Table I. List of mutant GTPases characterized in this study

Class: Mutational effect in the FtsY GTPase	FtsY mutation	Corresponding mutation in Ffh
I: Complex formation defective	G455W	ND
	E475K	ND
	K399A	ND
II: GTPase activation defective	N302A	Q109A
	R333A	R141A
	A335W	A143W
	A336W	A144W
	R386A	R194A
III: Both steps defective	T307A	ND

### Mutant FtsYs defective in reciprocal GTPase activation block the protein-targeting reaction

With the assay in hand, we tested the translocation efficiency of another class of FtsY mutants. Class II (activation-defective) mutants allow a stable SRP–FtsY complex to be assembled, but specifically block the reciprocal activation of both GTPase sites (Table I; Shan et al., 2004). The GTPase activity of these mutants was previously characterized in the context of a truncated version of FtsY, FtsY(47–497), in which the N-terminal 46 amino acids were removed to allow better expression and solubility of the protein. These amino acids are not important for FtsY's GTPase activity or for its interaction with SRP. Nevertheless, as the N-terminal A domain of FtsY (residues 1–196) has been implicated in its membrane association (Powers and Walter, 1997; Zelazny et al., 1997; Herskovits et al., 2001; Angelini et al., 2006), we reintroduced these mutations into full-length FtsY. However, cells harboring most of the class II FtsY mutants were sick and grew slowly, and only small quantities of these proteins were produced. Nevertheless, we succeeded in purifying sufficient quantities of full-length FtsY bearing two of the class II mutations (FtsY R386A and FtsY N302W; Table I). The other class II mutants (FtsY A335W, FtsY A336W, and FtsY R333A) could not be expressed, and were therefore only characterized in the context of FtsY(47–497) and compared with the wild-type FtsY(47–497) protein. In our assay, FtsY(47–497) exhibited only a twofold reduction in translocation efficiency compared with full-length FtsY (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200702018/DC1>). The mildness of the translocation defect exhibited by FtsY(47–497) may stem from the fact that only a small portion of the A domain of FtsY is removed in this construct and/or that our cotranslational targeting assay can only detect relatively large defects in protein targeting as discussed in the previous section. A recent study also suggests that the majority of the A domain of *E. coli* FtsY is not essential for the function of FtsY, as a truncated FtsY(196–497) construct containing only Phe196 in its A domain can rescue protein targeting and cell growth in vivo (Eitan and Bibi, 2004). It therefore seems reasonable that characterization of the mutants in this targeting assay yields meaningful results even in the context of the truncated FtsY(47–497).

All of the class II mutants compromise the efficiency of the translocation reaction, both in the context of full-length FtsY

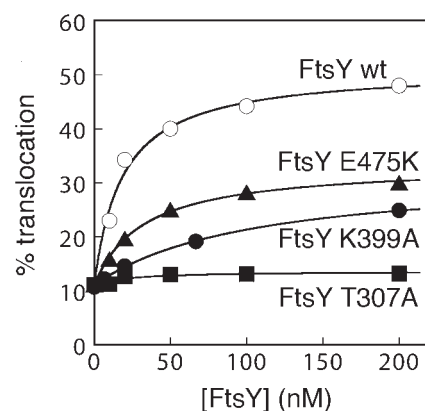


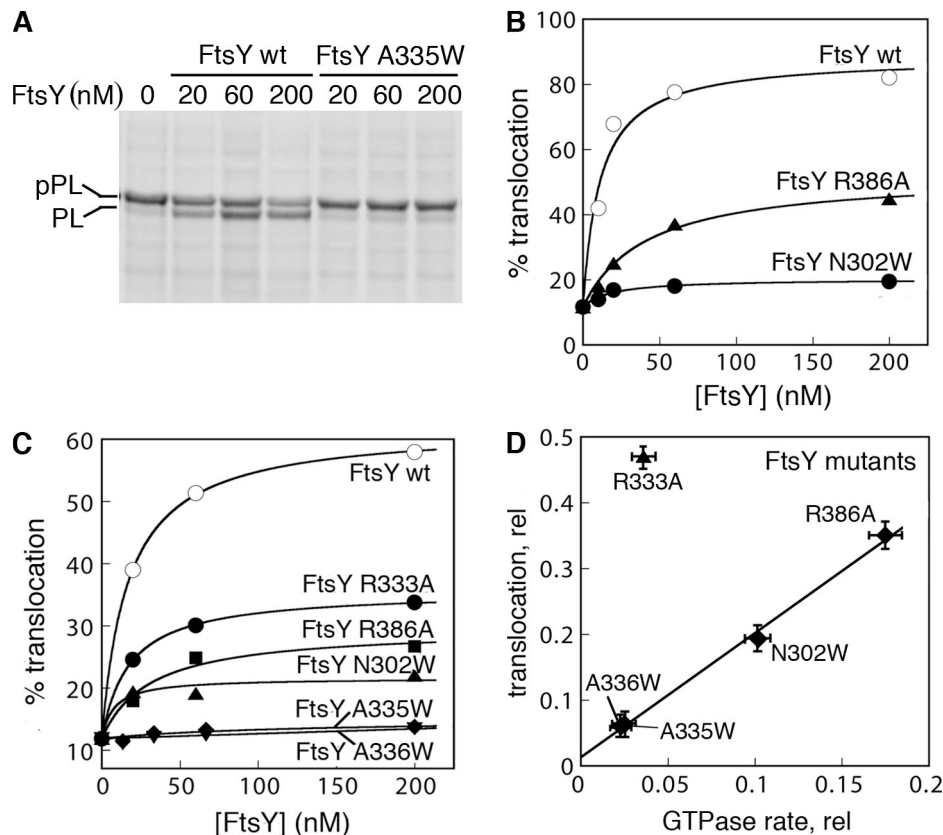
Figure 2. Calibration of the sensitivity and dynamic range of the cotranslational targeting assay using mutant GTPases known to block SRP–SRP binding. Translocation of pPL was performed in the presence of 150 nM SRP, 4 eq TKRM, and varying concentrations of FtsY as indicated. Representative data for three independent, side-by-side measurements are shown. The FtsY(47–497) construct was used and all the mutant proteins are derived from this construct.

(Fig. 3 B and Fig. S3 B) and the FtsY(47–497) protein (Fig. 3 C and Fig. S3 C). The translocation defect of each mutant compared with the respective wild-type protein is the same, within the margin of error, regardless of whether the mutations are introduced in the context of the full-length or truncated FtsY. Except for mutant FtsY R333A, a correlation can be found between the translocation defect of each mutant and the degree to which reciprocal GTPase activation is blocked in the mutant SRP–FtsY complex (Fig. 3 D). For example, one of the most severe mutants, FtsY A335W, which binds SRP with wild-type affinity but reduces the stimulated GTPase rate by >50-fold, almost completely blocks pPL translocation (Fig. 3, A and C). In contrast, the mutant FtsY R386A, which reduces the GTPase rate by sixfold, causes only a threefold reduction in translocation efficiency (Fig. 3, B–D). The deviation observed with mutant FtsY R333A (Fig. 3 D, triangle) potentially stems from the fact that, whereas the other mutants inhibit GTP hydrolysis primarily by blocking the conformational rearrangement that leads to GTPase activation, the FtsY R333A mutation also removes a key catalytic residue that directly participates in the chemical reaction (Egea et al., 2004). As discussed later, the rearrangement of the IBD loop is critical for protein translocation, whereas GTP hydrolysis is not (see Figs. 6 and 7 and Discussion). Therefore, the FtsY R333A mutant is more active in protein translocation than would be expected from its reduction in GTPase rate.

### Conserved IBD loops in both SRP and SR play essential roles in GTPase activation and in protein translocation

The data presented so far show that all of the class II FtsY mutants severely compromise the efficiency of protein targeting. Three of these mutations (FtsY R333A, FtsY A335W, and FtsY A336W) map to the highly conserved IBD loop. Because the IBD loop is conserved between the two GTPases and the catalytic interactions made by each IBD loop with the respective GTP molecule are highly symmetrical (Egea et al., 2004; Focia et al., 2004),





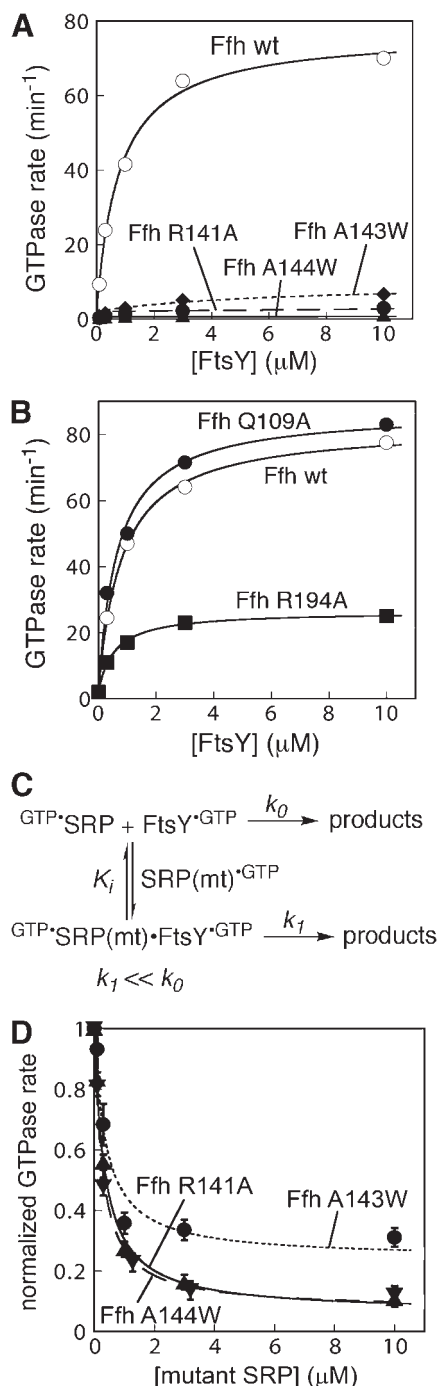
**Figure 3. Class II mutant FtsYs that block reciprocal GTPase activation in the SRP-FtsY complex exhibit severe translocation defects.** (A) The FtsY A335W mutant severely blocks protein targeting. The mutant and wild-type proteins are characterized in the context of the FtsY(47–497) construct. (B and C) Quantitation of the translocation defect of class II mutant FtsYs in the context of full-length FtsY (B) or the FtsY(47–497) construct (C). Representative data for three to five independent, side-by-side measurements are shown. (D) Correlation of the translocation activity of each FtsY mutant with its GTP hydrolysis rate from the SRP-FtsY complex. The translocation and GTPase activities of each mutant are normalized to those of the wild-type FtsY(47–497) at saturating protein concentrations and are averaged over three to five parallel measurements. The error bars represent the range of values observed.

we hypothesized that the residues in the IBD loop of the SRP GTPase also play a crucial role in two aspects: reciprocal GTPase activation and protein targeting. We therefore introduced the class II mutations, so far only characterized in FtsY, into the homologous positions of the SRP GTPase Ffh (Table I).

Mutant Ffh proteins were first characterized in terms of their basal GTPase cycles and their stimulated GTPase reaction upon interaction with FtsY. Most of the mutations do not affect the basal GTP binding and hydrolysis cycle of Ffh, except for Ffh R141A and Ffh A144W, which reduce the basal GTP hydrolysis rate by 8- and 20-fold, respectively (unpublished data). Notably, of the five class II mutants in FtsY, all three mutations in the IBD loop also substantially reduce the rate of stimulated GTP hydrolysis from the SRP-FtsY complex when introduced into homologous positions in Ffh (Fig. 4 A and Fig. S3 D). The effects of the other two mutations in this class are much milder when introduced into homologous positions in Ffh. The Ffh R194A mutation reduces the rate of stimulated GTP hydrolysis less than threefold, and the Ffh Q109A mutation has no effect on the stimulated GTPase reaction (Fig. 4 B and Fig. S3 E). Thus, there is a break in the functional symmetry of residues not residing in the IBD loop.

These results indicate that the most conserved and symmetrical feature between the two GTPases are the catalytic

interactions made by the IBD loops. In FtsY, the class II mutations in this loop can still allow a stable complex to assemble. To test if this is also true for the corresponding mutations in the IBD loop of Ffh, we used a slight modification of an inhibition assay previously developed (Shan et al., 2004) to determine the ability of each mutant SRP to inhibit the interaction of wild-type SRP with FtsY. This assay allowed us to selectively monitor complex formation between FtsY and the mutant SRPs. The conditions of the assay were designed so that in the absence of any mutant SRP as an inhibitor, a robust GTPase reaction mediated by wild-type SRP and FtsY was observed (Fig. 4 C,  $k_0$ ). Addition of mutant SRP, SRP(mt), which can form a complex with FtsY, will sequester the FtsY molecules into a less active SRP(mt)-FtsY complex ( $k_1 \ll k_0$ ), thus inhibiting the observed GTPase reaction. All three mutants are strong competitive inhibitors (Fig. 4 D), indicating that these mutant SRPs can form a strong complex with FtsY. The observed inhibition constants range from 260 to 390 nM for the three SRP mutants. However, because at least 300 nM FtsY needs to be present to allow a sufficient amount of GTPase reaction, and at least as much mutant SRP is needed to sequester all the FtsY molecules into the SRP(mt)-FtsY complex, the measured apparent inhibition constants represent an upper limit for the actual affinity of the mutant SRPs for FtsY.



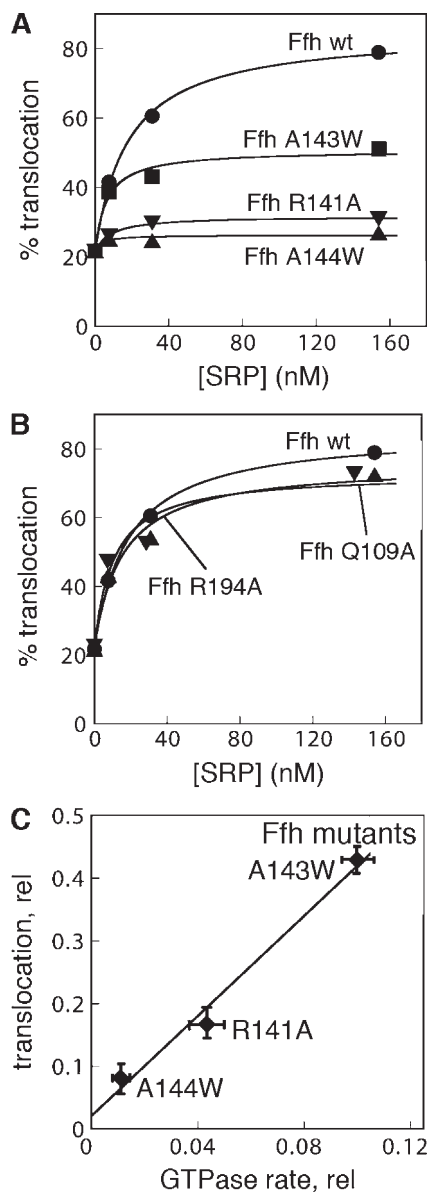
**Figure 4. Effect of mutations in the Ffh GTPase site on the reciprocally stimulated GTPase reaction between SRP and FtsY.** (A) Mutations in the IBD loop of Ffh substantially compromise reciprocal activation of GTP hydrolysis from the SRP–FtsY complex. Representative data for three independent, side-by-side measurements are shown. (B) Mutants Ffh Q109A and Ffh R194A do not substantially (less than threefold) compromise the formation and activation of the SRP–FtsY complex. Representative data for four independent, side-by-side measurements are shown. (C) Inhibition assay to measure the ability of the mutant SRPs (SRP(mt)) to form a complex with FtsY, as described in the text. (D) Ffh harboring mutations in the IBD loop are strong competitive inhibitors of wild-type SRP. The lines are fits of the data to inhibition curves and give apparent inhibition constants of 0.39, 0.27, and 0.35  $\mu\text{M}$  for Ffh A143W (●), Ffh A144W (▲), and Ffh R141A (▼), respectively. The data are averaged over three independent measurements and the error bars represent the range of values observed.

Thus mutations in the IBD loop of Ffh also result in the class II phenotype, with the mutant SRPs able to form a stable complex with FtsY but failing to efficiently activate GTP hydrolysis in the complex. Analogous to the results obtained with the FtsY that belong to this class, these class II SRP mutants also exhibit a substantial defect in mediating translocation of pPL (Fig. 5 A and Fig. S3 F). The translocation defect for each mutant correlates well with the amount of reduction in the stimulated GTP hydrolysis rates from the mutant SRP–FtsY complex (Fig. 5 C). In contrast, mutants Ffh Q109A and Ffh R194A (which map outside the IBD loop) exhibit no translocation defect (Fig. 5 B and Fig. S3 G). This is consistent with the observation that the Ffh Q109A mutation does not affect the activated GTPase reaction in the SRP–FtsY complex, and the Ffh R194A mutant has only a mild effect on GTPase activation (Fig. 4 B), and this small effect is not sufficient to manifest itself as a substantial translocation defect given the sensitivity of our targeting assay (see first section of Results).

Our analyses of the mutant GTPases indicate that blocking GTPase activation in the SRP–FtsY complex severely impairs protein translocation. This is surprising in light of the results from the pioneering studies by Wilson et al. (1988), which showed that a nonhydrolyzable GTP analogue, GMPPNP, can allow a single round of protein translocation to occur in the mammalian SRP system. To ensure that the translocation defect we observed with the mutant GTPases is not caused by the use of heterologous components, we reexamined the nucleotide requirement for translocation in our system. The cotranslational assay (Fig. 1 A) was inappropriate for this purpose, however, as GTP is also required for ongoing protein translation in addition to translocation. We therefore used an alternative assay in which a stalled RNC was generated by translation of a truncated mRNA that encodes the first 86 amino acids of pPL (pPL<sub>86</sub>). Nucleotides were removed from RNC<sub>pPL86</sub> by gel filtration chromatography (Wilson et al., 1988), and targeting of purified RNC<sub>pPL86</sub> to TKRM by bacterial SRP and FtsY was assayed in the presence of various nucleotides (Fig. 6 A). Both GTP and GMPPNP mediated efficient translocation of pPL<sub>86</sub>, as indicated by the production of PL<sub>56</sub> as pPL<sub>86</sub> was translocated across the microsomal membranes and processed by signal peptidase (Fig. 6 B). In contrast, in the presence of GDP or with no nucleotide added, no more than background levels of translocation were observed (Fig. 6, B and C). The class II mutant FtsYs described in the previous section still exhibit a large translocation defect in this posttranslational assay (unpublished data). These results confirm the conclusions by Wilson et al. (1988) and demonstrate that the translocation defect of the mutant GTPases arises from a block of the conformational rearrangements that lead to GTPase activation, rather than inhibition of the chemical reaction of GTP hydrolysis itself.

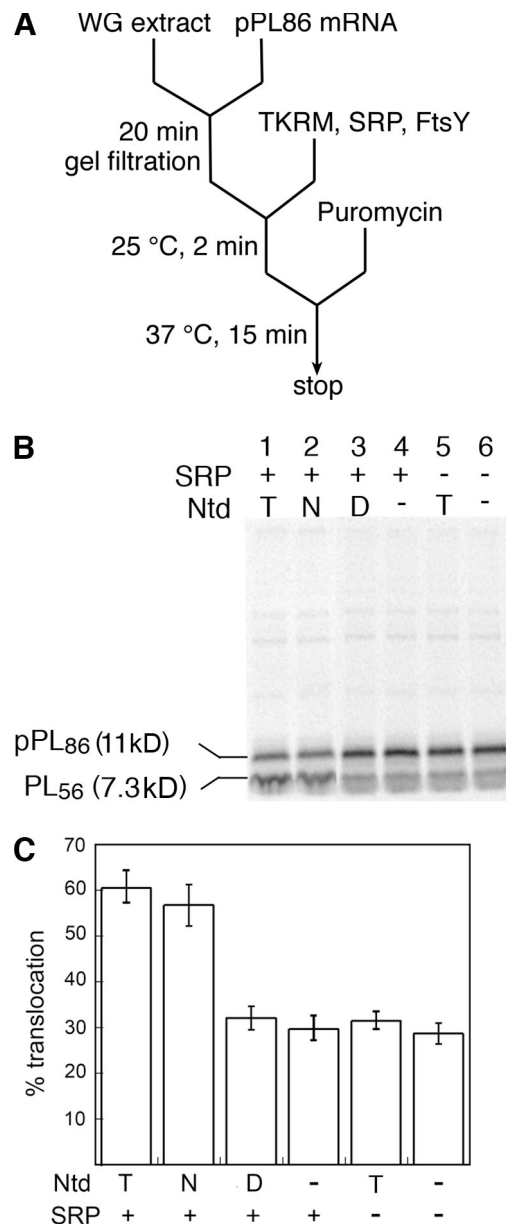
## Discussion

The two GTPases in SRP and SR use their GTPase cycles to regulate cotranslational targeting of proteins to membranes. However, the regulatory mechanism of SRP and SR GTPases



**Figure 5. Effect of mutations in Ffh on the efficiency of protein translocation.** (A) Mutations in the IBD loop of Ffh inhibit translocation of pPL. Representative data for three independent, side-by-side measurements are shown. (B) Mutants Ffh Q109A (▲) and Ffh R194A (▼) exhibit wild-type levels of translocation activity. Representative data for four independent, side-by-side measurements are shown. (C) Correlation of the translocation defect of Ffh mutants with their GTP hydrolysis rate from the SRP-FtsY complex. The translocation and GTPase activities of each mutant are normalized to those of wild-type Ffh at saturating protein concentrations and are averaged over three independent measurements. The error bars represent the range of values observed.

is a notable exception to the GTPase switch paradigm established for classical signaling GTPases. We have previously isolated different classes of mutant GTPases that block the binding and reciprocal activation between SRP and FtsY at specific stages (Shan et al., 2004). Analyses of these mutants reveal a series of discrete conformational rearrangements that occur during the interaction between SRP and FtsY, culminating in the reciprocal activation of GTP hydrolysis in both proteins. Here, we have used these mutants to examine the role of these



**Figure 6. Nucleotide requirement for protein translocation.** (A) Schematic of the posttranslational targeting assay using stalled RNC. (B and C) Effect of nucleotides on the efficiency of protein translocation. T, GTP; D, GDP; N, GMPPNP. The data are the mean of over two independent measurements and the error bars represent the range of values observed.

conformational changes in a complete, functional protein targeting reaction.

Fig. 7 summarizes the effect of the different classes of mutant GTPases on protein targeting and translocation. All of the mutant GTPases that compromise SRP-SR complex formation reduce the efficiency of protein targeting (Fig. 7 A, blue box). This result is consistent with the notion that formation of a stable SRP-SR complex is crucial for delivery of the cargo protein to the target membrane (Fig. 7 B, 2). Contrary to expectations based on a previous paper (Wilson et al., 1988), we found that the class II (activation-defective) mutants (Fig. 7 A, red box) also block efficient protein translocation when introduced into either FtsY or Ffh. The translocation defect correlates well

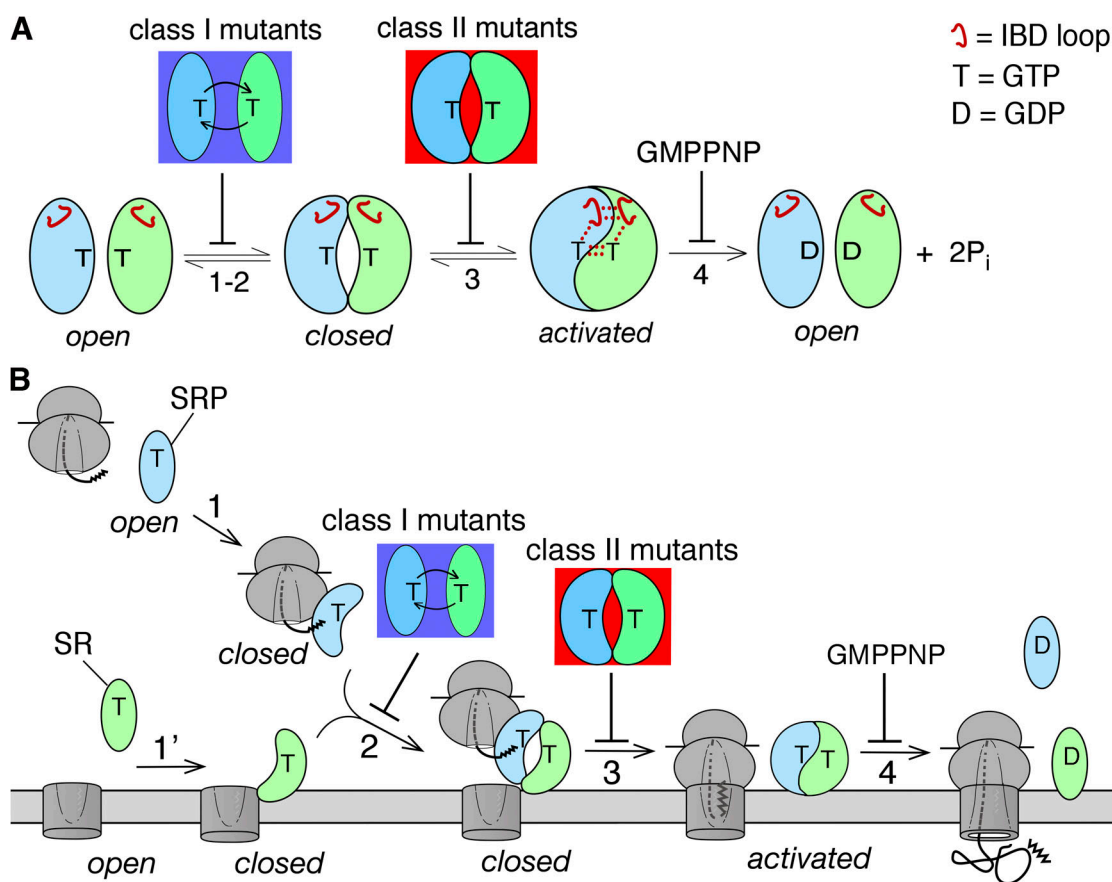


with the degree to which GTPase activation is inhibited by these mutants (Figs. 3 D and 5 C). In both GTPases, the class II mutations allow a stable SRP–SR complex to be assembled, but specifically inhibit the reciprocal activation of GTP hydrolysis in the complex.

The fact that these mutants block the protein translocation reaction is intriguing in light of the previous results obtained using GMPPNP (Wilson et al., 1988). Yet these seemingly contradictory results are easily reconciled by the fact that GMPPNP and the mutant GTPases inhibit the SRP–SR interaction cycle at different stages. GMPPNP is a good mimic of GTP that allows all or most of the conformational changes in the SRP–SR complex to occur, but blocks the chemical step of GTP hydrolysis caused by substitution of the  $\beta$ – $\gamma$  phosphate bridging oxygen with an imino group (Fig. 7 A). In contrast, the class II mutant GTPases block GTP hydrolysis at an earlier stage by inhibiting the conformational rearrangements that lead to activation of the two GTPase sites (Fig. 7 A, red box). Thus, although GTP hydrolysis is not, per se, required, the conformational changes upon the SRP–SR

interaction that lead to GTPase activation play a crucial role in the protein targeting reaction.

What structural changes in SRP and SR are responsible for mediating both GTPase activation and efficient targeting of the nascent protein? Analyses of the mutational effects suggest that movement of the IBD loop is the most crucial feature (Shan et al., 2004; this study). In both GTPases, mutations in this loop result in pronounced class II phenotypes and block protein translocation. The similar effects of these mutations in both GTPases are consistent with the symmetrical pattern of the interaction network formed between these loops and the GTP molecules bound at the respective active site (Egea et al., 2004; Focia et al., 2004). In contrast, the other two FtsY mutations in this class, when introduced into homologous positions in Ffh, do not substantially block reciprocal GTPase activation or the protein targeting reaction, indicating that the interactions made by these residues are less conserved and not symmetrical between the two GTPases. Therefore, an impairment to properly rearrange the IBD loop stands out as the likely cause leading to the functional targeting defects observed here.



**Figure 7. Mutant GTPases and GMPPNP block the SRP–SR interaction and the protein targeting reaction at distinct stages.** (A) Model for the effects of mutant GTPases and GMPPNP on SRP–SR binding and activation. (1–2) An open-to-closed conformational change in both SRP and SR is required to form a stable SRP–SR complex. This step is specifically inhibited by the class I mutants shown in the blue box. (3) Concerted docking of the IBD loops in both GTPases to form an activated complex. This step is specifically inhibited by the class II mutants shown in the red box. (4) GTP hydrolysis occurs from the activated SRP–SR complex and drives complex disassembly. This step is inhibited by GMPPNP. (B) Model for the effects of mutant GTPases and GMPPNP on the protein targeting reaction. (1 and 1') An open-to-closed conformational change occurs in SRP and SR upon binding to RNC and to the target membrane, respectively. (2) Complex formation between SRP and SR delivers the RNC to the membrane. This step is depicted as the target of inhibition by the class I mutant GTPases. Alternatively, these mutants could inhibit the open-to-closed conformational change that precedes complex formation (1 and 1'), but for simplicity this alternative scenario is not depicted. (3) Conformational rearrangements in the SRP–SR complex activate GTP hydrolysis and unload the RNC from the SRP to the membrane translocation channel. This step is inhibited by the class II mutants. (4) GTP hydrolysis drives complex disassembly and recycling of the SRP and SR components. This step is inhibited by GMPPNP.

How do the activation-defective GTPase mutants block the protein targeting reaction? We consider it most likely that these mutants block the cargo-unloading step, at which the RNC is released from SRP and transferred to the translocon embedded in the membrane (Fig. 7 B, 3). Cargo unloading has to occur after a stable SRP–SR complex is formed, but before GTP hydrolysis is activated to drive complex disassembly. Other steps in the targeting reaction are less likely to be a target of these mutant GTPases: binding of SRP to the RNC (Fig. 7 B, 1) should not be affected by mutations in FtsY, which does not participate in cargo recognition. Formation of the SRP–SR complex, which mediates delivery of cargo to the membrane surface (Fig. 7 B, 2), is also unlikely because this class of mutants has been shown to form stable SRP–FtsY complexes. Finally, hydrolysis of GTP to drive the dissociation and recycling of the SRP components (Fig. 7 B, 4) is unlikely because our assay monitors a single round of protein targeting and any defect on this step would not be observed. A role of the SRP–SR interaction in facilitating cargo release is also suggested by the recent cryo-EM structures of the RNC–SRP and RNC–SRP–SR complexes (Halic et al., 2004, 2006). Comparison of the two structures shows that, upon binding of SR to the RNC–SRP complex, the electron density of the GTPase domains of both SRP and SR is no longer visible, although the other domains of SRP and SR can be identified and remain close to the RNC. Thus the interaction with the SR induces structural rearrangements that change the way SRP is positioned at the ribosome exit tunnel.

Collectively, both the biochemical and structural characterizations suggest that the concerted rearrangements that occur upon formation of the SRP–SR complex serve dual purposes. First, movement of the IBD loops into close proximity to the bound GTP activates GTP hydrolysis in the complex that sets the stage for subsequent disassembly and recycling of the SRP components. Second, these movements trigger (directly or indirectly) the switch of SRP from the cargo-binding mode to the cargo-release mode, and thus help drive the transfer of the nascent chain from SRP to the translocon. In this way, cargo transfer and GTPase activation are effectively coupled to each other to ensure the maximum efficiency by which cargo protein is delivered to the translocation channel on the target membrane. Further, by using the conformational change for GTPase activation to trigger cargo release, GTP hydrolysis could also be used by the SRP to improve the fidelity of the protein targeting reaction akin to kinetic proofreading mechanisms used by elongation factor GTPases (Rodnina and Wintermeyer, 2001), although no concrete evidence in support of this notion is currently available. Most importantly, our results imply that bringing SRP and SR together in a complex, and thereby juxtaposing the RNC and the translocon at the membrane surface, is not sufficient to initiate transfer of the nascent chain from SRP to the translocon. Rather, for SRP and SR to exert their roles as molecular matchmakers, an active cargo-unloading step has to take place that requires an elaborate conformational rearrangement within the complexed GTPase modules of SRP and SR.

## Materials and methods

### Materials

WG translation extract was obtained from Promega. Microsomal membranes from dog pancreas were prepared by J. Miller (University of California, San Francisco, San Francisco, CA) according to published procedures (Walter and Blobel, 1983) and were treated with high salt and partial trypsin digestion to generate TKRM as described previously (Gilmore et al., 1982a; Walter and Blobel, 1983; Andrews et al., 1989). The in vitro transcription plasmid for pPL was provided by E. Powers (University of California, Davis, Davis, CA). The expression and purification of Ffh and FtsY have been described previously (Powers and Walter, 1997; Peluso et al., 2001). Mutant Ffh and FtsY proteins were constructed using the QuickChange mutagenesis protocol (Stratagene). Mutant Ffh and FtsY were purified using the same procedures as those for wild-type proteins. [<sup>35</sup>S]methionine and γ-[<sup>32</sup>P]GTP were obtained from GE Healthcare.

### Protein-targeting assays

The cotranslational protein-targeting assay was described in detail in the text (Fig. 1 A) and in a previous paper (Powers and Walter, 1997). Post-translational protein-targeting assay of pPL<sub>86</sub> (Fig. 6 A) was performed with slight modifications of the procedures used by Wilson et al. (1988). Stalled RNCs containing pPL<sub>86</sub> were generated by in vitro translation using the WG translation extract. After completion of translation, nucleotides were removed from the RNC using a 1-ml Sephacryl S-200 gel filtration column (Sigma-Aldrich; Wilson et al., 1988). Fractions in the void volume containing purified RNC were pooled and incubated with TKRM for 2 min at 25°C. 2 mM puromycin was added and the reaction mixture was incubated at 37°C for 15 min; this step releases the pPL<sub>86</sub> nascent chain from the ribosome to allow for its translocation across the membrane and cleavage by the signal peptidase. The reaction was then analyzed by 15% SDS-PAGE.

The translocation efficiency of each mutant GTPase was measured in parallel with that of the wild-type protein, and these comparative measurements were repeated three to five times. Most of the figures presented in this paper show a representative measurement performed in parallel for all the proteins. In general, the absolute translocation efficiency for each protein can vary up to 30% from day to day and depends on several factors such as the amount and purity of TKRM and the quality of [<sup>35</sup>S]methionine. Nevertheless, these are systematic rather than random errors, and the translocation efficiency of the mutant relative to the wild-type protein, measured in side-by-side experiments, is highly reproducible and independent of the aforementioned factors, with deviations of <10%.

### GTPase assays

GTP hydrolysis reactions were performed and analyzed as described previously (Peluso et al., 2001). The use of the GTPase assay to measure the basal GTPase activity of Ffh, the stimulated GTPase reaction between SRP and FtsY, and the affinity between a mutant GTPase and its binding partner have been described in detail previously (Peluso et al., 2001; Shan et al., 2004).

### Online supplemental material

Fig. S1 shows that the targeting and translocation of pPL occurs on a faster time scale than completion of protein synthesis. Fig. S2 shows that FtsY(47–497) is reduced by about half in translocation of pPL compared with full-length FtsY. Fig. S3 and Tables S1–S7 show additional data for repetitions of the experiments shown in Figs. 2–5. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200702018/DC1>.

We thank Ted Powers for help in reconstituting the heterologous protein targeting reaction.

This work was supported by National Institutes of Health grant GM 32384 to P. Walter and career awards from the Burroughs Wellcome Fund and the Camille and Henry Dreyfus Foundation to S. Shan. P. Walter is an investigator at the Howard Hughes Medical Institute.

Submitted: 5 February 2007

Accepted: 1 July 2007

## References

- Andrews, D.W., L. Lauffer, P. Walter, and V.R. Lingappa. 1989. Evidence for a two-step mechanism involved in assembly of functional signal recognition particle receptor. *J. Cell Biol.* 108:797–810.
- Angelini, S., D. Boy, E. Schiltz, and H.-G. Koch. 2006. Membrane binding of the bacterial signal recognition particle receptor involves two distinct binding modes. *J. Cell Biol.* 174:715–724.

- Bourne, H.R., D.A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature*. 349:117–127.
- Connolly, T., and R. Gilmore. 1989. The signal recognition particle receptor mediates the GTP-dependent displacement of SRP from the signal sequence of the nascent polypeptide. *Cell*. 57:599–610.
- Connolly, T., and R. Gilmore. 1993. GTP hydrolysis by complexes of the signal recognition particle and the signal recognition particle receptor. *J. Cell Biol.* 123:799–807.
- Connolly, T., P.J. Rapiejko, and R. Gilmore. 1991. Requirement of GTP hydrolysis for dissociation of the signal recognition particle from its receptor. *Science*. 252:1171–1173.
- Egea, P.F., S. Shan, J. Napetschnig, D.F. Savage, P. Walter, and R.M. Stroud. 2004. Substrate twinning activates the signal recognition particle and its receptor. *Nature*. 427:215–221.
- Eitan, A., and E. Bibi. 2004. The core *Escherichia coli* signal recognition particle receptor contains only the N and G domains of FtsY. *J. Bacteriol.* 186:2492–2494.
- Focia, P.J., I.V. Shepotinovskaya, J.A. Seidler, and D.M. Freymann. 2004. Heterodimeric GTPase core of the SRP targeting complex. *Science*. 303:373–377.
- Freymann, D.M., R.J. Keenan, R.M. Stroud, and P. Walter. 1997. Structure of the conserved GTPase domain of the signal recognition particle. *Nature*. 385:361–364.
- Freymann, D.M., R.J. Keenan, R.M. Stroud, and P. Walter. 1999. Functional changes in the structure of the SRP GTPase on binding GDP and Mg<sup>2+</sup>GDP. *Nat. Struct. Biol.* 6:793–801.
- Gilman, A.G. 1987. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* 56:615–649.
- Gilmore, R., G. Blobel, and P. Walter. 1982a. Protein translocation across the endoplasmic reticulum: I. Detection in the microsomal membrane of a receptor for the signal recognition particle. *J. Cell Biol.* 95:463–469.
- Gilmore, R., P. Walter, and G. Blobel. 1982b. Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. *J. Cell Biol.* 95:470–477.
- Halic, M., T. Becker, M.R. Pool, C.M.T. Spahn, R.A. Grassucci, J. Frank, and R. Beckmann. 2004. Structure of the signal recognition particle interacting with the elongation-arrested ribosome. *Nature*. 427:808–814.
- Halic, M., M. Gartmann, O. Schlenker, T. Mielke, M.R. Pool, I. Sinning, and R. Beckmann. 2006. Signal recognition particle receptor exposes the ribosomal translocon binding site. *Science*. 312:745–747.
- Herskovits, A.A., A. Seluanov, R. Rajsbaum, C.M. ten Hagen-Jongman, T. Henrichs, E.S. Bochkareva, G.J. Philips, F.J. Probst, T. Nakae, M. Ehrmann, J. Lührink, and E. Bibi. 2001. Evidence for coupling of membrane targeting and function of the signal recognition particle (SRP) receptor FtsY. *EMBO Rep.* 2:1040–1046.
- Jagath, J.R., M.V. Rodnina, G. Lentzen, and W. Wintermeyer. 1998. Interaction of guanine nucleotides with the signal recognition particle from *Escherichia coli*. *Biochemistry*. 37:15408–15413.
- Jagath, J.R., M.V. Rodnina, and W. Wintermeyer. 2000. Conformational changes in the bacterial SRP receptor FtsY upon binding of guanine nucleotides and SRP. *J. Mol. Biol.* 295:745–753.
- Keenan, R.J., D.M. Freymann, R.M. Stroud, and P. Walter. 2001. The signal recognition particle. *Annu. Rev. Biochem.* 70:755–775.
- Koch, H.-G., T. Hengelage, C. Neumann-Haufelin, J. MacFarlane, H.K. Hoffschulte, K.-L. Schimz, B. Mechler, and M. Müller. 1999. In vitro studies with purified components reveal signal recognition particle (SRP) and SecA/SecB as constituents of two independent protein-targeting pathways of *Escherichia coli*. *Mol. Biol. Cell*. 10:2163–2173.
- Koch, H.-G., M. Moser, K.-L. Schimz, and M. Müller. 2002. The integration of YidC into the cytoplasmic membrane of *Escherichia coli* requires the signal recognition particle, SecA and SecYEG. *J. Biol. Chem.* 277:5715–5718.
- Miller, J.D., H. Wilhelm, L. Gierasch, R. Gilmore, and P. Walter. 1993. GTP binding and hydrolysis by the signal recognition particle during initiation of protein translocation. *Nature*. 366:351–354.
- Mitin, N., K.L. Rossman, and C.J. Der. 2005. Signaling interplay in Ras superfamily function. *Curr. Biol.* 15:R563–R574.
- Montoya, G., C. Svensson, J. Lührink, and I. Sinning. 1997. Crystal structure of the NG domain from the signal recognition particle receptor FtsY. *Nature*. 385:365–368.
- Padmanabhan, S., and D.M. Freymann. 2001. The conformation of bound GMPPNP suggests a mechanism for gating the active site of the SRP GTPase site. *Structure*. 9:859–867.
- Peluso, P., D. Herschlag, S. Nock, D.M. Freymann, A.E. Johnson, and P. Walter. 2000. Role of 4.5S RNA in assembly of the bacterial signal recognition particle with its receptor. *Science*. 288:1640–1643.
- Peluso, P., S. Shan, S. Nock, D. Herschlag, and P. Walter. 2001. Role of SRP RNA in the GTPase cycles of Ffh and FtsY. *Biochemistry*. 40:15224–15233.
- Pool, M.R., J. Stumm, T.A. Fulga, I. Sinning, and B. Dobberstein. 2002. Distinct modes of signal recognition particle interaction with the ribosome. *Science*. 297:1345–1348.
- Powers, T., and P. Walter. 1995. Reciprocal stimulation of GTP hydrolysis by two directly interacting GTPases. *Science*. 269:1422–1424.
- Powers, T., and P. Walter. 1997. Co-translational protein targeting catalyzed by the *Escherichia coli* signal recognition particle and its receptor. *EMBO J.* 16:4880–4886.
- Rodnina, M.V., and W. Wintermeyer. 2001. Ribosome fidelity: tRNA discrimination, proofreading and induced fit. *Trends Biochem. Sci.* 26:124–130.
- Shan, S., and P. Walter. 2003. Induced nucleotide specificity in a GTPase. *Proc. Natl. Acad. Sci. USA*. 100:4480–4485.
- Shan, S., R. Stroud, and P. Walter. 2004. Mechanism of association and reciprocal activation of two GTPases. *PLoS Biol.* 2:e320.
- Valent, Q.A., D.A. Kendall, S. High, R. Kusters, B. Oudega, and J. Lührink. 1995. Early events in preprotein recognition in *E. coli*: interaction of SRP and trigger factor with nascent polypeptides. *EMBO J.* 14:5494–5505.
- Valent, Q.A., P.A. Scotti, S. High, J.W.L. de Gier, G. von Heijne, G. Lentzen, W. Wintermeyer, B. Oudega, and J. Lührink. 1998. The *Escherichia coli* SRP and SecB targeting pathways converge at the translocon. *EMBO J.* 17:2504–2512.
- Walter, P., and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum III. Signal recognition protein (SRP) causes signal sequence-dependent and site-specific arrest of chain elongation that is released by microsomal membranes. *J. Cell Biol.* 91:557–561.
- Walter, P., and G. Blobel. 1983. Preparation of microsomal membranes for cotranslational protein translocation. *Methods Enzymol.* 96:84–93.
- Walter, P., and A.E. Johnson. 1994. Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* 10:87–119.
- Walter, P., I. Ibrahim, and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum I. Signal recognition protein (SRP) binds to in-vitro-assembled polysomes synthesizing secretory protein. *J. Cell Biol.* 91:545–550.
- Wilson, C., T. Connolly, T. Morrison, and R. Gilmore. 1988. Integration of membrane proteins into the endoplasmic reticulum requires GTP. *J. Cell Biol.* 107:69–77.
- Zelazny, A., A. Seluanov, A. Cooper, and E. Bibi. 1997. The NG domain of the prokaryotic signal recognition particle receptor, FtsY, is fully functional when fused to an unrelated integral membrane polypeptide. *Proc. Natl. Acad. Sci. USA*. 94:6025–6029.